ACTIN IN ERYTHROCYTE GHOSTS

AND ITS ASSOCIATION WITH SPECTRIN

Evidence for a Nonfilamentous

Form of These Two Molecules *In Situ*

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ABSTRACT

Actin was isolated from erythrocyte ghosts. It is identical to muscle actin in its molecular weight, net charge, ability to polymerize into filaments with the double helical morphology, and its decoration with heavy meromyosin (HMM). When erythrocyte ghosts are incubated in 0.1 mM EDTA, actin and spectrin are solubilized. Spectrin has a larger molecular weight than muscle myosin. When salt is added to the EDTA extract, a branching filamentous polymer is formed. However, when muscle actin and the EDTA extract are mixed together in the presence of salt, the viscosity achieved is less than the viscosity of the solution if spectrin is omitted. Thus, spectrin seems to inhibit the polymerization of actin. If the actin is already polymerized, the addition of spectrin increases the viscosity of the solution, presumably by cross-linking the actin filaments. The addition of HMM or trypsin to erythrocyte ghosts results in filament formation *in situ*. These agents apparently act by detaching erythrocyte actin from spectrin, thereby allowing the polymerization of one or both proteins to occur. Since filaments are not present in untreated erythrocyte ghosts, we conclude that erythrocyte actin and spectrin associate to form an anastomosing network beneath the erythrocyte membrane. This network presumably functions in restricting the lateral movement of membrane-penetrating particles.

The molecular biology of membranes is an area of extremely active research at the present time, as can be attested to by the large number of reviews and books published on this subject over the past few years (see Singer, 1974, for references). The erythrocyte membrane has been a favorite source of experimental material because (*a*) it is readily available, (*b*) it is relatively homogeneous; and (*c*) it can be prepared without contamination of other membrane fragments or cytoplasm. The literature concerning the proteins in this membrane has been extensively reviewed; no less than 10 reviews have appeared in the last two years (see Steck, 1974).

About 20–25% of the total protein in erythrocyte membranes is made up of a pair of proteins called spectrin (bands 1 and 2 on SDS polyacrylamide gels) by Marchesi and Steers (1968). These proteins have molecular weights of 250,000 and 220,000 (Fairbanks et al., 1971) and appear to function, at least in part, in constraining the lateral

motion of membrane-penetrating particles (Elgsaeter and Branton, 1974). When spectrin is isolated from ghosts by incubating the ghosts with EDTA (Fairbanks et al., 1971) or low salt (Marchesi and Steers, 1968; Elgsaeter and Branton, 1974), a second protein component is invariably isolated with it, so-called band 5. This protein has a molecular weight of 43,000 and comprises about 4% of the total membrane protein.

Over the years there has been considerable speculation on the possibility that an actomyosin system exists in the erythrocyte ghost (Guidotti, 1972). This speculation was initiated by Ohnishi (1962), who added muscle myosin to a water extract of an acetone powder of erythrocyte ghosts and demonstrated by viscometry that the myosin interacted with a protein in the extract. This interaction could be inhibited with ATP; in the parlance of muscle biochemists, it showed ATP sensitivity. More recently, Marchesi and Palade (1967 a) demonstrated that when ghosts were trypsinized in the presence of ATP and Mg^{++}, filaments appeared which had the double helical morphology of F-actin. Unfortunately, the interpretation of these discoveries was confused by a subsequent report (Marchesi and Steers, 1968) which showed that, although similar filaments could be polymerized by the addition of Mg^{++} to the proteins obtained by dialysing ghosts for 24 h against distilled water to which mercaptoethanol was added, antibodies to this solution would not react with muscle actin. Instead, the antibodies cross-reacted only with ghost membranes. Thus, Marchesi and Steers (1968) concluded that spectrin was not actin. On polyacrylamide gels of the extract, they found only one major band which they called spectrin. (Actually, there is a minor component on their gels as well which will be shown to be actin in this report.)

The idea that actomyosin was associated with erythrocyte membranes persisted, however, based on the relative molecular weights of bands 1, 2 (in the range of myosin) and 5 (similar to muscle actin) and on the fact that band 2 seems to be a weak Ca^{++}-dependent ATPase (Marchesi et al., 1970; Rosenthal et al., 1970). The other evidence in support of this idea is either too weak to consider seriously or is published only as a "personal communication" (see Steck, 1974) and therefore is impossible to evaluate.

Considering the volume of literature on erythrocyte membranes, it is surprising, and in fact disturbing, that no one has looked rigorously into the question concerning the actual existence of actin and myosin in erythrocyte membranes. Perhaps data are lacking in this area because, under the usual conditions of fixation, filaments are not attached to the inner surface of the erythrocyte membrane. Instead of filaments there appears to be an amorphous material which may in fact be composed of fine, short elements about 200 Å in length (Zelander and Ericsson, 1965; Rosenthal et al., 1970). As will be demonstrated shortly, these are probably spectrin monomers or dimers. Only under conditions such as trypsinization (Marchesi and Palade, 1967 a), long-term dialysis (Marchesi and Steers, 1968) or extraction of ghosts with EDTA (Marchesi and Palade, 1967 b) followed by the addition of salt, can one demonstrate the presence of filaments of any appreciable length. This is true irregardless of the technique used to examine erythrocyte ghosts: thin sectioning, negative staining (Yu et al., 1973), or freeze-fracturing (Tillack and Marchesi, 1970). The report of Tillack and Marchesi (1970) using freeze-fracturing is most revealing in this regard. These investigators fixed ghosts in the presence of F-actin from skeletal muscle in order to be able to identify the outer surface of the membrane. In an etched preparation the muscle actin filaments can readily be identified by the characteristic double helical substructure. Since muscle actin filaments can easily be identified attached to the outer surface of the ghost membrane by this technique, there is no reason why actin filaments, or, for that matter, filaments of any kind, would not also be resolved on the inner surface of the ghost membrane if they existed. Since these investigators did not see filaments within the ghost or attached to the inner surface of the erythrocyte membrane, their evidence demonstrates that filaments do not exist there in situ. Despite this evidence there has been a fantastic amount written about fibrous or filamentous material attached to the inner surface of erythrocyte ghosts (see Elgsaeter and Branton, 1974; Steck, 1974; Rosenthal et al., 1970; Marchesi, 1974; Singer, 1974). These reports, which conflict with available evidence, apparently result from the fact that filaments can be induced to form if ghosts are mistreated in certain ways, i.e., trypsinization, extraction with EDTA, or dialysis against water for several days followed by the addition of salt. Likewise, filaments will form in vitro if salt is added to a solution of spectrin (Clarke, 1971; Rosenthal et al., 1970).

The purpose of this paper is to first demonstrate

that actin is indeed present in erythrocyte ghosts. We will then present evidence that will lead us to a new interpretation of the state of actin and spectrin in the erythrocyte membrane. We will suggest that spectrin is not in the form of filaments *in situ* as has been concluded by many investigators (see Elgsaeter and Branton, 1974; Steck, 1974; Rosenthal et al., 1970; Marchesi, 1974; Singer, 1974), but instead forms an anastomosing network beneath the erythrocyte membrane in combination with monomeric actin.

MATERIALS AND METHODS

Preparation of Ghosts

Recently outdated human whole blood (the blood was used within the same week that it was outdated) or whole blood that was newly collected was obtained from local hospitals. To prepare ghosts we followed the procedure of Fairbanks et al. (1971). The blood was washed four times in large volumes of 0.15 M NaCl and 5 mM phosphate buffer at pH 8.0 to remove platelets, serum, and leukocytes. Examination of the washed blood with the light microscope revealed no contaminating platelets. Ghosts were prepared by lysing the cells with 5 mM phosphate buffer and then washing them repeatedly to remove all traces of hemoglobin.

Preparation of an Acetone
Powder of Ghosts

Purified ghosts were extracted with at least 10 vol of acetone at 4°C. They were washed three times with fresh acetone. The powder was air dried overnight and stored in a dessicator at 0°C.

Preparation of Muscle Protein

Muscle actin was prepared from an acetone powder of rabbit skeletal muscle by extracting 1 g of powder with 25 ml of water for 30 min at 0°C. The residuum was removed by centrifugation (10,000 × g for 10 min); the supernate was filtered.

Myosin and heavy meromyosin (HMM) were prepared by standard procedures (see Tilney et al., 1973). Subfragment 1 (S_1) was prepared in two ways. The first method followed the procedure of Lowey et al. (1969) in which insoluble papain was employed. Digestion was stopped with iodoacetic acid which, as stated by Smith and Kimmel (1960), alkylates the sulfhydryl groups of the enzyme and completely destroys its activity. The inactivated papain was removed by centrifugation. More recently, we have used soluble papain instead. As before, digestion was stopped by iodoacetic acid. The S_1 was then isolated from light meromyosin (LMM) by low salt precipitation of the LMM. The S_1 was purified by

fractionation with ammonium sulfate (55%). Both the S_1 and HMM were stored at −20°C in 50% glycerol.

Viscosity

Viscosity determinations were made with an Ostwald-type capillary viscometer at 22 ± 0.1°C. The flow time for water was 26 s.

Gel Electrophoresis

5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) were run using 25 mM Tris glycine at pH 8.3 or the phosphate buffer system of Weber and Osborn (1969) as the running buffer. Skeletal muscle actin and myosin served as protein standards. Bromphenol blue was used as the tracking dye, and the gels were stained with Coomassie brilliant blue. Protein standards, whole ghosts and the EDTA extract of ghosts (see Fairbanks et al., 1971) were dissolved in 1% SDS in 2.5 mM Tris glycine at pH 8.3 or 10 mM phosphate buffer at pH 6.8. Samples were reduced in 1% mercaptoethanol and boiled for 2 min.

5% polyacrylamide gels in 8 M urea were also run. The samples, which included muscle actin and the EDTA extract of ghosts, were reduced and acetylated before the runs. The running buffer was 25 mM Tris glycine at pH 8.3.

Electron Microscopy

NEGATIVE STAINING: A drop of solution to be examined by negative staining was added to either a lightly carbonized collodion-coated grid or to a "holey" grid (Huxley and Zubay, 1960). The solution remained on the grid for periods of time ranging from 5 s to 2 min and then 0.5% unbuffered uranyl acetate was added as the negative stain. The excess stain was removed with filter paper. For S_1 or HMM binding, the solution containing the proteins to be tested was added to an equal volume of S_1 or HMM (0.5–2 mg/ml). The solution was allowed to stand at room temperature for a few minutes and then a drop was added to a grid and negatively stained.

FIXATION, EMBEDDING, AND THIN SECTIONING: Erythrocyte ghosts that had been treated with trypsin, S_1, HMM, or both S_1 and trypsin, were fixed in 2% glutaraldehyde from a 50% stock, (Fisher Scientific Co., Fair Lawn, N. J.; in another group of experiments, from an 8% stock, Electron Microscopy Sciences, Fort Washington, Pa.) in a 0.05 M phosphate buffer at pH 7.5 for 1–2 h, washed in buffer, and postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4°C. They were rinsed in distilled water twice and, before dehydration, were stained with 0.5% uranyl acetate for 2 h at 0°C. They were then dehydrated and embedded in araldite. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum II ultramicrotome and stained with uranyl acetate and lead citrate. They were viewed with a Philips 200 electron microscope.

HMM or S_1 Binding In Situ

Purified ghosts were incubated in S_1 or HMM. The final concentration of S_1 or HMM was about 2 mg/ml. In one group of experiments ghosts were glycerinated in 50% glycerol in the presence of HMM and 10 mM phosphate buffer at pH 7.5 for 30 min at 37°C. In two other groups of experiments ghosts were incubated in S_1 at 4°C for 12 h with or without glycerol. There was no difference in the results, regardless of what temperature incubation was carried out at or whether or not glycerol was added.

In a final group of experiments, we added trypsin (10 μg/ml) to purified ghosts for 15 min at 37°C. The trypsin was then inactivated with an excess of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.).

RESULTS

Actin in Erythrocyte Ghosts

As described by Fairbanks et al. (1971), when erythrocyte ghosts are run by SDS gel electrophoresis, about nine major bands are present. These bands have been numbered according to their electrophoretic mobilities. Of particular interest to this report are bands 1 and 2 (spectrin) and band 5.

FIGURE 2   Polyacrylamide gels containing 8 M urea. Left gel, EDTA extract. Bands 1 and 2 (spectrin) and band 5 are indicated. Right gel, skeletal muscle actin. Note that a band from the EDTA extract has the same net charge as actin on these urea gels.
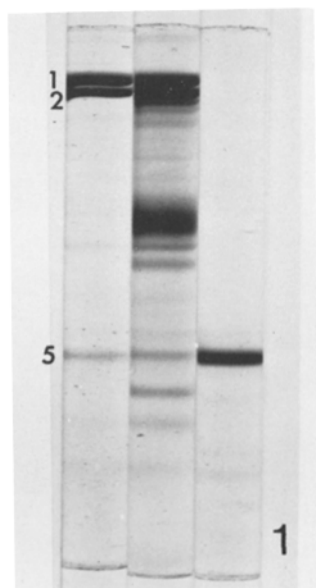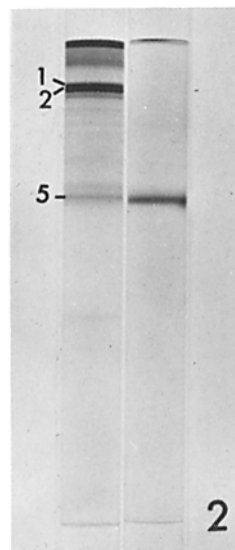
FIGURE 1   SDS gel electrophoresis. Purified erythrocyte ghosts were extracted with 0.1 mM EDTA and the supernate was run (left gel); three bands appear, bands 1 and 2 (spectrin) and band 5. The center gel illustrates the proteins of erythrocyte ghosts. The right gel represents purified skeletal muscle actin. Note that band 5 has the same molecular weight as muscle actin.

Band 5 has a molecular weight indistinguishable from that of muscle actin (Fig. 1). When ghosts are extracted with 0.1 mM EDTA (Fairbanks et al., 1971) and the residual membranes removed by centrifugation, the supernate contains bands 1, 2 and 5 plus some minor contamination from the other components (Fig. 1). If this EDTA extract is reduced, acetylated and run on polyacrylamide gels containing 8 M urea, two major bands appear. One of these is spectrin (bands 1 and 2), and the other is band 5 (Fig. 2). Band 5 has the same mobility as muscle actin, indicating that band 5 and actin have an identical net charge and molecular weight.

When an acetone powder of erythrocyte ghosts is extracted with water and 0.1 M KCl is added, filaments, each about 50 Å in diameter, can be seen in negatively stained preparations. These filaments resemble actin morphologically; they show the typical double helical structure. If S_1 is added to this extract and the solution negatively stained, the filaments show the characteristic arrowhead pattern of decorated actin filaments (Fig. 3). The periodicity of the arrowheads is about 375 Å. The yield of actin from the ghosts is difficult to estimate because of the small amount of acetone powder obtainable from ghosts, beginning with

100 ml of blood. In addition, if S_1 and salt are added to the EDTA extract, decorated filaments can be demonstrated by negative staining (Fig. 4), although the clarity of the decorations is not so

FIGURE 3 An acetone powder of purified erythrocyte ghosts was extracted with water. KCl was added to the extract to 0.1 M, then S_1 was added. The filaments illustrated show the characteristic "arrowhead pattern" of decorated actin filaments. The arrow indicates the polarity of the filaments in this negatively stained preparation. × 115,000.

obvious as that seen when actin is extracted from the acetone powder. This is due to the tremendous amount of unpolymerized spectrin which forms the background and tends to adhere to some of the filaments. When we added S_1 to the spectrin polymer, the polymer did not decorate. More will said about this polymer later.

Thus, actin appears to be present in erythrocytes on the basis of its characteristic molecular weight, net charge, ability to form filaments with a double helical morphology, and, most importantly, the ability to be decorated with S_1 or HMM.

Since there have been reports that myosin may be similar to spectrin (Guidotti, 1972), we coelectrophoresed the EDTA extract which contained the spectrin and erythrocyte actin with skeletal muscle actomyosin (Fig. 5). Whereas muscle actin and erythrocyte actin comigrate, myosin does not comigrate with either band 1 or band 2 of spectrin. In fact, myosin has a lower molecular weight than either band 1 or 2. This indicates not only that spectrin does not have a molecular weight identical to that of myosin, but also that neither spectrin band can be a proteolytic fragment of myosin.

Spectrin: Its Monomeric and Polymeric Forms

When the EDTA extract (about 0.09 mg/ml) is examined by negative staining in the absence of salt, the grid is covered by small, curved elements that generally take the form of a "C" (Fig. 6). Each C is approximately 250 Å in length. Since the bulk of this extract contains bands 1 and 2 (spectrin), band 5 contributing about one-sixth to
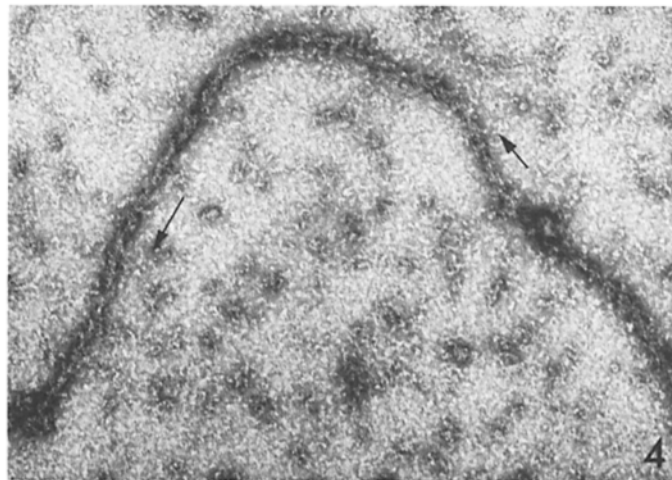

FIGURE 4 S_1 was added to the EDTA extract which was then negatively stained. The arrows indicate the polarity of a decorated filament. In the background is a large amount of unpolymerized spectrin. × 65,000.

one-fifth of the staining on an SDS or urea gel, the Cs must be unpolymerized spectrin. If salt is added to the extract and the solution negatively stained 10–15 min later, the grids are covered by a filamentous polymer (Fig. 7). The diameters of the filaments vary from about 40 to 120 Å and the filaments, unlike F-actin, are not double helical. Generally, the filaments tend to align themselves parallel to each other and thus form large bundles. Under these conditions adjacent filaments seem to be connected by strands of similar material or to be branched. A similar polymer has been described by Rosenthal et al. (1970), when 2 mM $CaCl_2$ is added to the EDTA extract of ghosts.

Thin Sections of Erythrocyte Ghosts

As has been demonstrated by many investigators the erythrocyte ghost membrane in transverse section (Fig. 8) consists of two dense lines separated by an intervening less dense space. On the cytoplasmic surface is an amorphous, almost cotton-like material. In certain places this fuzz appears as short, thin elements. The diameters of these structures are about 30 Å and their lengths seldom exceed 200 Å. These filaments can best be seen in grazing sections. The bulk of the material, however, appears amorphous. The interior of the ghosts is empty.

Effect of S_1 or HMM on Erythrocyte Ghosts

The interior of ghosts which have been incubated with S_1 or HMM with or without glycerina-

tion is filled with a filamentous material (Fig. 9). Most of the filaments appear to have material attached to their surfaces. It is very difficult to identify good examples of "decorated" filaments formed by an interaction between an actin filament and S_1. There are regions on some of the filaments that have periodic projections on them, as if they might have been decorated. Some of the filaments seem to attach to the plasma membrane. The
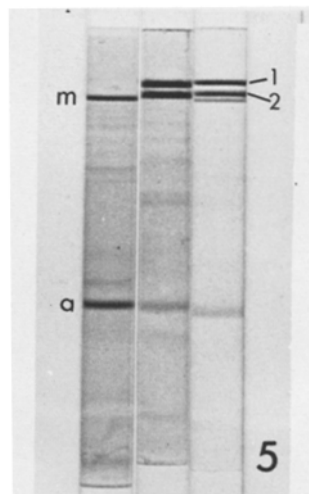

FIGURE 5 SDS gel electrophoresis of muscle actomyosin (left gel), and the EDTA extract of erythrocyte ghosts (center gel). When the EDTA extract and actomyosin are run in the same gel (right gel), it is clear that the actin (a) in the two gels comigrates but that myosin (m) migrates at a faster mobility than spectrin (bands 1 and 2).
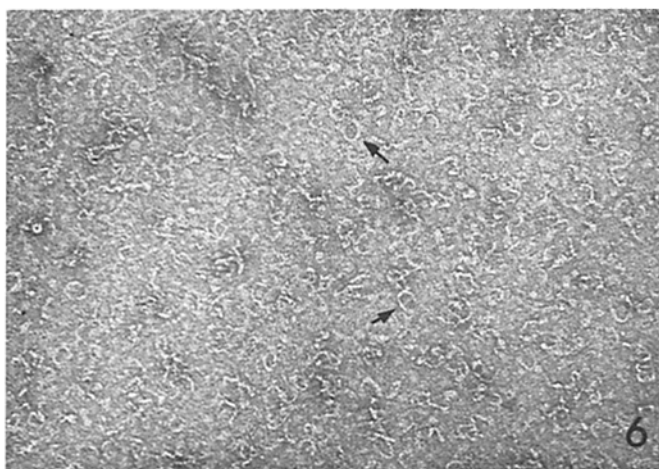

FIGURE 6 Negatively stained preparation of the EDTA extract containing unpolymerized spectrin. The extract consists of elements which resemble Cs. Some of these are indicated by the arrows. × 105,000.
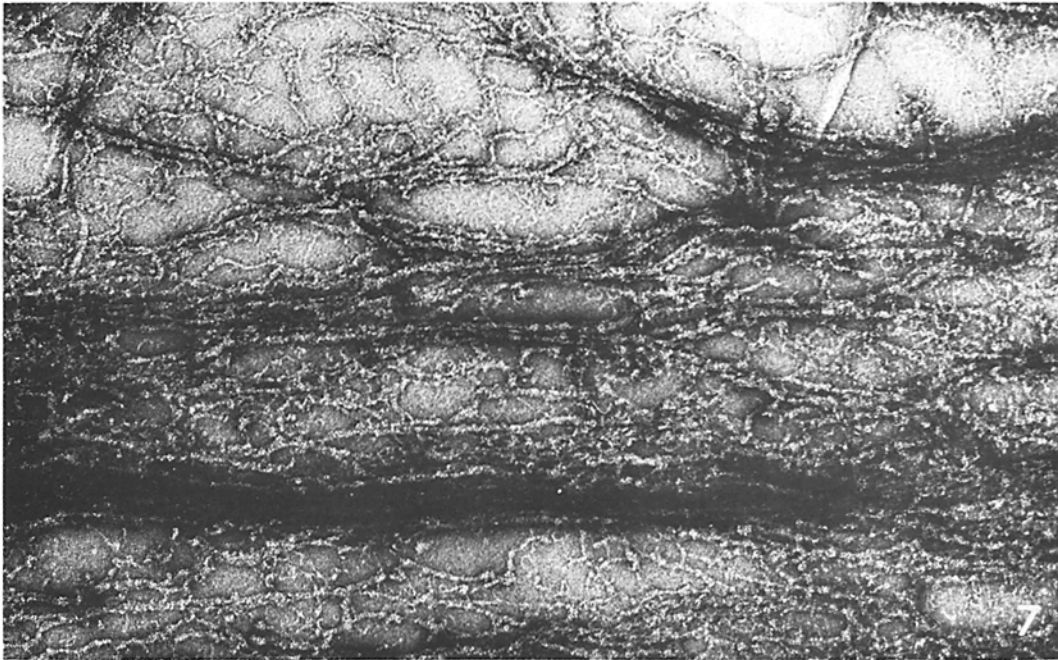
FIGURE 7 When salt (50 mM KCl and 1 mM MgCl₂) is added to the EDTA extract, the spectrin will polymerize. The polymer is illustrated in this negatively stained preparation. × 75,000.
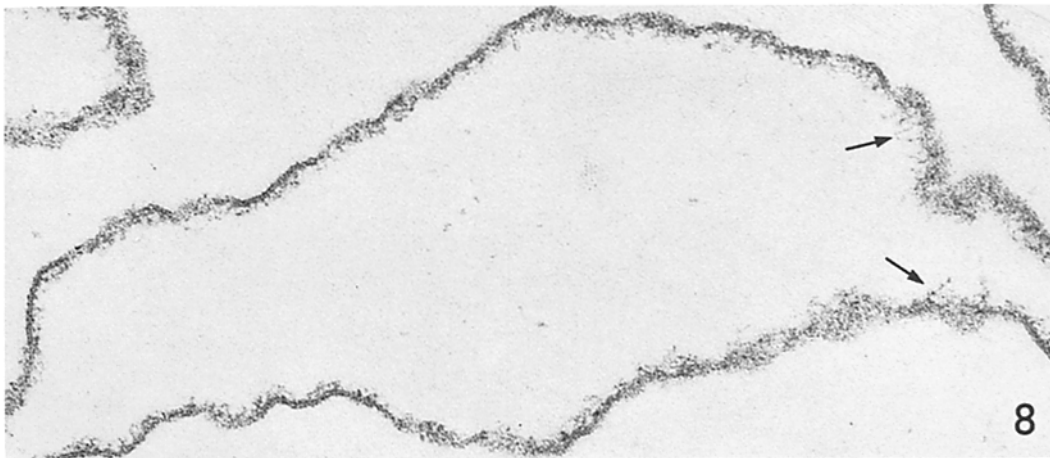

FIGURE 8 Thin section through a portion of an erythrocyte ghost. On the cytoplasmic surface of the plasma membrane of the ghosts is an amorphous material. Short filaments about 200 Å in length are indicated by the arrows. No material is present within the lumen. × 100,000.

amorphous fuzz that was attached to the inner surface of the untreated erythrocyte membrane has largely disappeared from the treated ghosts. Presumably, it is now located within the ghost proper. Considering the amount of actin present in SDS gels of erythrocyte ghosts (about 4% of the total protein), there are too many filaments present in the interior of the ghost to be accounted for by band 5 alone. Since the fuzz has disappeared from the cytoplasmic surface of the membrane, much of the filamentous material within the ghost can be attributed to bands 1 and 2 as well as to band 5.

Perhaps some of these filaments are the branching spectrin polymers seen in the negatively stained image.

Effect of Trypsin on
Erythrocyte Ghosts

As was originally demonstrated by Marchesi and Palade (1967 a), if ghosts are treated with trypsin in the presence of ATP and salt, filaments which resemble F-actin in morphology appear in negatively stained preparations. When we repreated this experiment and examined the ghosts in thin sections, within the interior we found large numbers of filaments (Fig. 10). The morphology of these filaments resembles that of the actin fila-

ments that are found attached to isolated amoeba membranes (Pollard and Korn, 1973). As in the case where ghosts were incubated in the presence of S_1, the amorphous fuzz formerly on the cytoplasmic surface of the plasma membrane has disappeared. Some of this material appears as filaments within the interior of the ghost, the rest must have been digested.

When ghosts are first digested with trypsin and then treated with S_1, very few filaments are present. Those filaments that are present have material associated with them which gives the impression that they are decorated. Why there is such a large reduction in the number of filaments in this experiment compared with the experiment
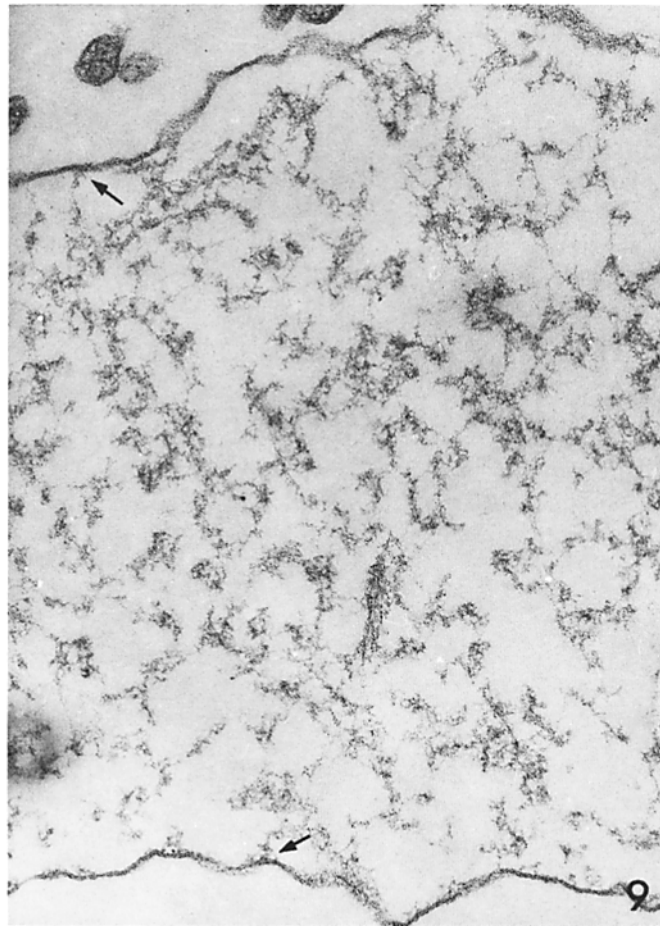
FIGURE 9   Thin section through a portion of an erythrocyte ghost that has been incubated in the presence of S_1. Within the lumen are large numbers of filaments. Some of these filaments appear to have material attached to them as if decorated. Others appear to be attached to the plasma membrane (see arrows). Note that the cytoplasmic surface of the plasma membrane has no amorphous material attached to it. × 70,000.
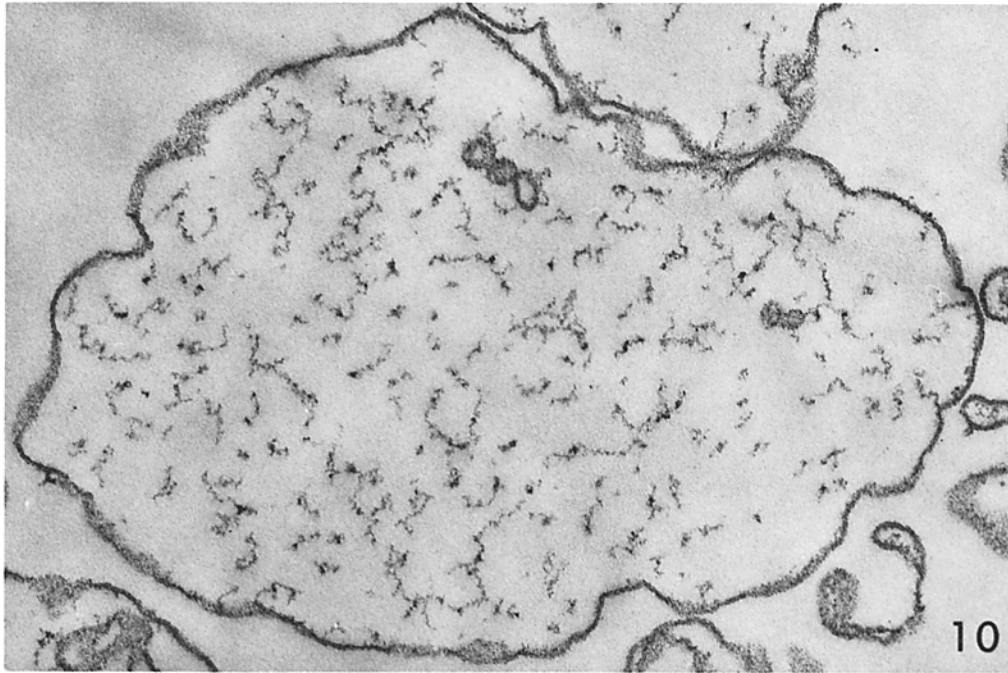
FIGURE 10 Thin section through a portion of an erythrocyte ghost which has been incubated in the presence of trypsin. Filaments are now present within the ghost. Note that the cytoplasmic surface of the plasma membrane is bare; the amorphous material has disappeared. × 75,000.

with trypsin alone may be explained by the diffusion of material from inside the ghosts. Trypsin may make holes in the ghosts so that when ghosts are incubated with S_1 for either 30 min at 37°C or overnight at 4°C, much of the material which once was attached to the cytoplasmic surface of the membrane is able to diffuse away.

Polymerization of Spectrin and Actin In Vitro

If the EDTA extract (about 0.09 mg/ml) is treated with salt (1 mM $MgCl_2$, 40 mM KCl), there is essentially no increase in the viscosity of the solution, due to the low concentration of actin and spectrin in this solution (Fig. 11, curve a). When salt (1 mM $MgCl_2$, 40 mM KCl) is added to monomeric muscle actin (G-actin) (about 0.3 mg/ml), there is a rapid increase in the viscosity of the solution (Fig. 11, curve c), related, of course, to the polymerization of G-actin into F-actin. (If 0.1 mM EDTA is added to the solution of muscle G-actin, the polymerization is unaffected.) However, if we add the EDTA extract which contains spectrin to monomeric muscle actin and then add salt, the

resultant mixture does not achieve as high a viscosity as the muscle actin alone (Fig. 11, curve b). (For this experiment we kept the concentrations of actin and the EDTA extract the same as they were for curves a and c.) Alternatively, if we take the same amount of muscle actin used in the previous two experiments (curves b and c of Fig. 11), polymerize it and then add the EDTA extract, a higher viscosity is achieved (Fig. 11, curve d) than when the muscle actin alone is measured. The most obvious interpretation of these results is that the spectrin, which is the main component of the EDTA extract, inhibits the polymerization of muscle G-actin, possibly by capping polymerizing filaments. If the actin is already in the F state, however, the spectrin acts to link the actin filaments together. The negatively stained image of these solutions is consistent with this interpretation. When the solution represented by curve b was negatively stained, we could see a lot of disorganized material (Fig. 12 a). On the other hand, when the solution from curve d was examined (Fig. 12 b), filaments were observed as well as some disorganized material. The disorganized material frequently appeared to be plastered over the

length of the filament. No disorganized material was present in the solution of actin by itself (curve c). Similar curves were obtained using light scattering at 90°.

DISCUSSION

In this report we have demonstrated that actin is present in erythrocyte ghosts. This assertion is based upon actin's molecular weight, its net charge, its ability to polymerize into filaments with the typical double helical structure of actin, and most important, its ability to form the characteristic arrowhead complexes seen when actin is combined with HMM or S_1. Secondly, we have shown that both erythrocyte actin and spectrin can polymerize in vitro to form filamentous polymers when salt is added to the monomeric material. Yet in thin sections through erythrocyte ghosts, filaments cannot be demonstrated; one does see, however, fine linear elements about 200 Å in length, the approximate length of our unpolymerized spectrin. And, finally, we have demonstrated that when salt is added to a solution containing muscle G-actin and spectrin, the viscosity of this solution is much less than one would expect in the absence of spectrin. Similar results were obtained from the light scattering observations. The most reasonable explanation for this result is that the spectrin is reducing the polymerization of the actin by inhibiting the elongation of the filaments. Consistent with such an interpretation is the negatively stained image.

We recognize, of course, that the results obtained by viscometry are open to several interpretations. We have, therefore, repeated these observations using light scattering and have obtained very similar results. Criticism can be directed against this technique as well, but since both give similar curves, we feel somewhat reassured. Clearly, many more experiments must be carried out to show that actin and spectrin bind in vitro. These experiments are in progress. We have described our experiments, although preliminary at this time, because the implications of an association between actin and spectrin have tremendous significance in the maintenance of not only the erythrocyte membrane but other membranes as well.

These observations and several more which we will discuss below lead us to a new interpretation of how actin and spectrin exist in situ. Since spectrin appears to inhibit the polymerization of actin in vitro and since no filaments appear to be present in vivo unless the cells are subjected to nonphysiological conditions, we suspected not only that spectrin might inhibit the polymerization of actin, but also that actin might inhibit the polymerization of spectrin. Thus, actin and spectrin together may form a mesh of unpolymerized material on the cytoplasmic surface of the lipid bilayer. Instead of these materials existing as a filamentous layer beneath the erythrocyte membrane as has been suggested repeatedly in the literature (Guidotti, 1972; Fig. 12 of Elgsaeter and Branton, 1974; Steck, 1974), we believe that the actin and spectrin form an anastomosing framework like a net woven
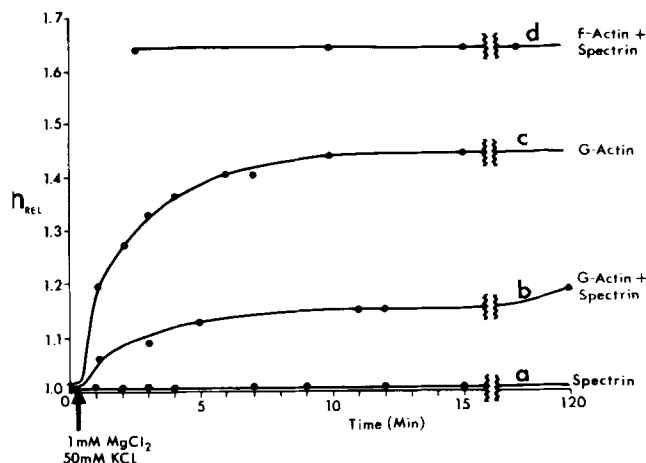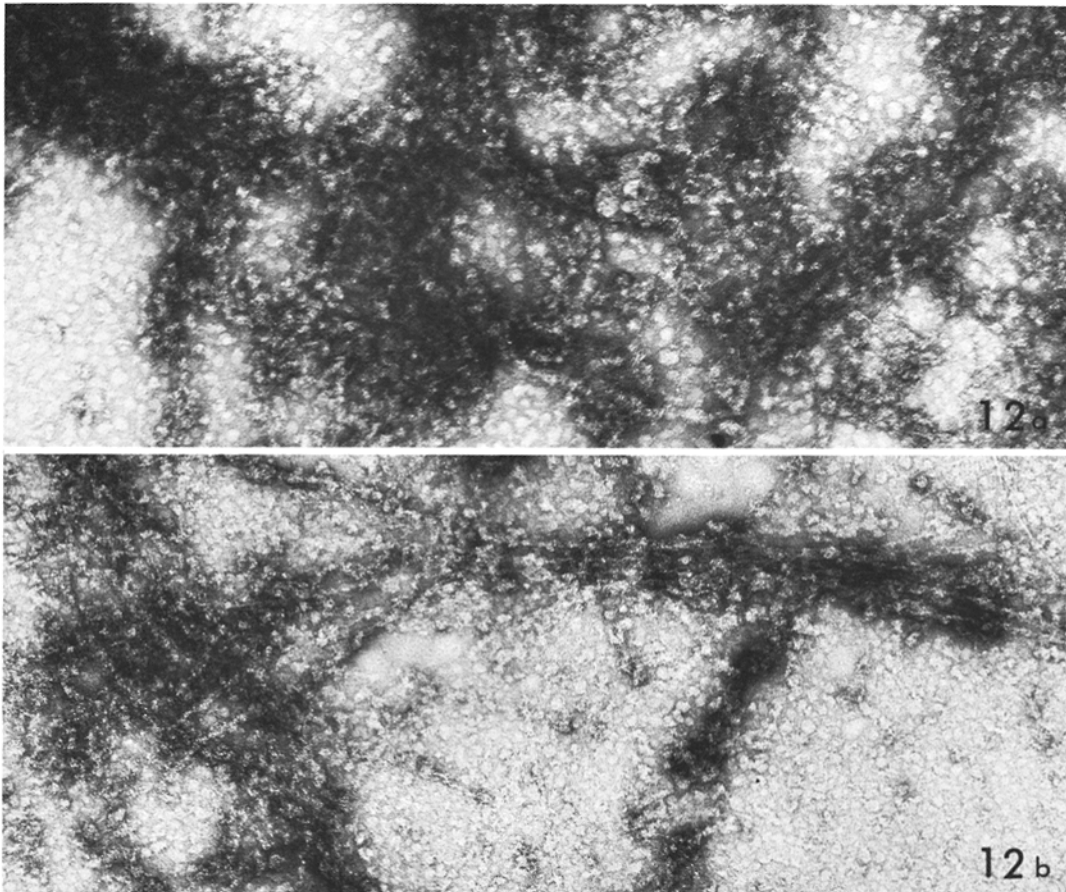

FIGURE 11   Viscometry of spectrin (the EDTA extract) and muscle actin at intervals after the addition of salt. The concentration of the spectrin is approximately 0.09 mg/ml, that of the muscle actin 0.3 mg/ml.

FIGURE 12 *a* Negatively stained image of the solution illustrated in curve *b* of Fig. 11, 6 min after the addition of salt. × 60,000.

FIGURE 12 *b* Negatively stained image of the solution illustrated in curve *d* of Fig. 11, 60 min after the addition of salt. × 60,000.

by a myopic fisherman (not too well ordered) where the spectrin subunits (a combination of bands 1 and 2, see Clarke, 1971) and actin subunits make up individual lines of the net. Consistent with this idea are, of course, our experiments in which ghosts are incubated in the presence of HMM or trypsin. Under both conditions filaments appear in the interior of the ghost at the expense of the fuzz which was formerly attached to the cytoplasmic surface of the membrane. Our interpretation of these experiments is as follows. If either actin or spectrin is partially or totally removed from the membrane by these agents (the spectrin by digestion, the actin by its polymerization when HMM is added), the remaining material is free to polymerize. (We should mention that actin is notoriously

trypsin-insensitive, while spectrin is rather sensitive to trypsin [Steck et al., 1971].) Furthermore, from these results, the viscometric data, and from the fact that, if one extracts spectrin, band 5 (actin) invariably is present in the solution as well (see Fairbanks et al., 1971; Steck and Yu, 1973; Yu et al., 1973), it seems reasonable that spectrin and actin may bind to each other in vivo. Moreover, one would predict the molar ratio between actin and spectrin to be 1:1. Spectrin has a molecular weight of 470,000, yet is made up of two components, 250,000 and 220,000 mol wt, respectively. Since each component of spectrin has a molecular weight about five times that of band 5 (actin) and comprises about 20–25% of the total erythrocyte protein, whereas band 5 is only about 4% (see

Elgsaeter and Branton, 1974; Steck, 1974), the molar ratio of band 5 to the combination of bands 1 and 2 is about 1:1 as predicted.

In our negatively stained preparations of un-polymerized spectrin we find a large population of small curled elements, each of which measures about 250 Å in length. These elements are presumably "monomeric" spectrin. Similar short filaments are seen attached to the ghost membrane *in situ* (Rosenthal et al., 1970; Zelander and Ericsson, 1965; and this report). The size of this unit is at variance with the size of spectrin reported by Clarke and Griffith (1972). These investigators report that the basic unit of spectrin is 2,100 Å in length. We presume that this unit must be an oligomer of the small elements discribed in this report. When the solution containing spectrin and actin (the EDTA extract) is treated with salt (Mg^{++} and K^+) it polymerized to form a branching filamentous polymer. The diameter of these filaments (Fig. 7) is extremely variable and the polymer appears to branch. Thus, spectrin appears to be capable of associating either end to end or side to side, or both, in the presence of salt to form an anastomosing network beneath the erythrocyte membrane.

Additional information is available which is consistent with this arrangement of spectrin and actin in ghost membranes. It further demonstrates that a spectrin-like molecule and actin may be present in many cell types. For example, we (Tilney, 1974; Tilney, 1975) demonstrated recently that a large amount of unpolymerized actin is present in echinoderm sperm before the acrosomal reaction. During the formation of the acrosomal process this actin polymerizes explosively. Of particular interest to this report is that the actin in the untreated sperm is associated with a pair of proteins whose molecular weights are similar to those of bands 1 and 2 (spectrin). Furthermore, if either HMM, S_1, or trypsin is added to demembranated sperm, the actin will polymerize to form filaments. We were able to show that brief trypsinization did not affect the molecular weight of actin, while the spectrin-like proteins were rapidly digested. In the sperm, then, we appear to have a situation homologous to that of actin and spectrin in the erythrocyte ghosts, that is, the actin is kept from polymerizing by spectrin-like proteins. If these proteins are removed by digestion or displaced from their binding to sperm actin by HMM, then the actin is competent to polymerize as is the case in the erythrocyte. There is one

distinction, however; the concentration of actin relative to that of spectrin is strikingly different in each of these two systems. This seems to be related to the presumed function of the spectrin-like molecules in each system. In the sperm the spectrin-like protein apparently functions to sequester the actin in a localized region of the cell in a non-filamentous state, whereas in the ghosts, because of the great excess of spectrin relative to the amount of actin (on a weight basis), the actin might function in keeping the spectrin unpolymerized, thus preventing the lateral translation of membrane-penetrating molecules.

Although we have looked for spectrin-like proteins only in echinoderm sperm and erythrocytes, when we were examining two other systems (developing myoblasts and chondroblasts) for another purpose we found a pair of bands whose electrophoretic mobility was similar to that of the bands in the erythrocyte ghosts and in sperm. More recently, Hartwig and Stossel (1975) examined macrophages and found a spectrin-like protein that binds to actin. This protein has a molecular weight similar to that of band 2 of erythrocyte spectrin. Furthermore, actin has been shown to be associated with the plasma membrane fraction of 3T3 and HeLa cells (Gruenstein et al., 1975). In HeLa cell membranes there is a pair of bands with the approximate electrophoretic mobility of spectrin. All of these reports lead us to speculate that spectrin and actin might be common to many cell membranes. The fact that actin is associated with the membranes is particularly intriguing when one considers that most "motile" processes in cells, such as cytokinesis, microspike formation, the acrosomal reaction, etc., take place in conjunction with membranes.

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